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Growth of *Moraxella catarrhalis* with Human Transferrin and Lactoferrin: Expression of Iron-Repressible Proteins without Siderophore Production

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***Moraxella* (*Branhamella*) *catarrhalis*, a mucosal pathogen closely related to *Neisseria* species, is a prominent cause of otitis media in young children and lower respiratory tract infections in adults. In this study, we investigated whether *M. catarrhalis* can compete for iron bound to human transferrin or human lactoferrin in a manner similar to that utilized by *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Our studies demonstrated that *M. catarrhalis* obtains iron from these serum carrier proteins and also maintains growth with ferric nitrate in vitro. Furthermore, we report that when *M. catarrhalis* is grown under iron-limited conditions, the bacteria express new outer membrane proteins that are not detected in membranes of organisms cultured in an iron-rich environment. We have shown that these are iron-repressible proteins since they are not induced by other environmental stresses and the expression of these proteins is repressed when a source of iron is provided for iron-limited bacteria. The iron-repressible proteins are expressed in the absence of any detectable siderophore production. These iron-repressible proteins may be important for the acquisition and utilization of iron in vivo, which could allow *M. catarrhalis* to colonize and survive on human mucosal surfaces.**

Moraxella (*Branhamella*) *catarrahalis* is a gram-negative diplococcal bacterium which has steadily emerged as an important human pathogen. The organism causes acute otitis media and sinusitis in infants and young children (4). The incidence of infections caused by this organism has risen over the past decade such that *M. catarrhalis* is now the third leading cause of otitis media, behind *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (9, 10). It is now estimated that 80% of children will have had at least one episode of otitis media by the age of 3, and 20% of these infections will be caused by *M. catarrhalis* (4, 16).

In adults, *M. catarrhalis* has also been implicated as a significant cause of lower respiratory tract infections in patients with chronic obstructive pulmonary disease (2, 18). These infections usually provoke acute exacerbations of chronic obstructive pulmonary disease (6, 10). In addition, *M. catarrhalis* occasionally causes severe infections such as septicemia, endocarditis, and meningitis in immunocompromised hosts (2, 4, 6).

Despite many studies describing membrane components of *M. catarrhalis*, little is known about potential virulence factors. The outer membrane protein (OMP) patterns of many strains of *M. catarrhalis* are similar (1, 16). Previous studies have identified conserved OMPs CopB or B2 and C/D, although the function of these proteins remains undefined (16, 17, 19). Antibodies to the CopB protein enhance the clearance of *M. catarrhalis* from the lungs of mice inoculated with this organism (7). More recently, Helminen et al. have demonstrated that the expression of the CopB protein may be involved in serum resistance and may also be important for survival of *M. catarrhalis* in these animals (8).

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In this report, we describe the first studies which demonstrate that *M. catarrhalis* expresses specific proteins when grown under iron-limiting conditions. Previously reported studies show that at least two of the iron-repressible proteins in our present study are expressed in vivo (7, 8, 23). This report also demonstrates that *M. catarrhalis* can utilize human lactoferrin and transferrin as a source of iron in the absence of any detectable siderophore production. Our data suggest that *M. catarrhalis* iron transport functions similarly to that of the other mucosal pathogens, such as *H. influenzae* and the pathogenic *Neisseria* species.

MATERIALS AND METHODS

Bacteria. Four strains of *M. catarrhalis* were kindly provided by Timothy Murphy. *M. catarrhalis* 4223 was isolated from the middle ear of a child with otitis media in Buffalo, N.Y. Strains 56 and 1084 were isolated from the sputum of adults with lower respiratory tract infections in Johnson City, Tenn., and Birmingham, England. *M. catarrhalis* 25240 was obtained from the American Type Culture Collection.

Preparation of growth media. To determine whether *M. catarrhalis* expressed iron-repressible proteins, the organisms were grown in the Chelex (Sigma)-treated, defined medium (CDM) of Morse and Bartenstein (15), as modified by West and Sparling (24). The defined medium was iron depleted by using Chelex-100 and prepared as described previously (24). This medium was called CDM 0.

The initial growth experiments used ferric nitrate as the only iron source. This medium was prepared by adding ferric nitrate to CDM medium at a final concentration of 100 μM. This medium was referred to as CDM 100.

Growth conditions. *M. catarrhalis* was inoculated onto CDM 0 agar plates and grown in a 5% CO_2 atmosphere at 37°C overnight. Organisms from these plates were used to inoculate a 40-ml CDM 0 broth culture, and the flasks were placed in a

Gyrotory-water bath shaker (New Brunswick Scientific) at 37°C with constant agitation of 160 rpm. After 18 h, fresh CDM 0 and CDM 100 broth cultures were inoculated with equal amounts of *M. catarrhalis* as determined by optical density at 600 nm (OD_{600}). The cultures were returned to the water bath shaker, and growth was monitored by measuring the OD_{600} at 1-h intervals.

In the experiments evaluating the effect of temperature on *M. catarrhalis*, the cultures were prepared exactly as described above, but they were maintained in the shaking water bath at 40°C.

Saturation of iron-binding proteins. Iron-poor, human transferrin (98% pure) and lactoferrin (90% pure) were purchased from Sigma Chemical Co. These proteins were saturated with iron by using ferric citrate as described previously (24). The percentages of iron saturation for each carrier protein used in the growth experiments were 49% for lactoferrin and 53% for transferrin.

Growth with transferrin and lactoferrin. The initial broth culture for these experiments contained *M. catarrhalis* 25240 inoculated into CDM 0 as described above. The iron-saturated transferrin and lactoferrin were evaluated for their ability to support growth at the following concentrations: 1.25, 2.5, 5.0, and 10.0 μM. Parallel cultures of CDM 0 and CDM 100 were included as controls for these experiments. All cultures were maintained at 37°C with constant agitation. Growth was monitored by measuring OD_{600} as described above.

Siderophore determinations. Broth culture supernatants from *M. catarrhalis* 25240, grown in CDM 0, were filter sterilized and analyzed for the presence of siderophores or iron-binding compounds by using the previously described method of Schywn and Neilands (22).

OMP preparations. OMPs were prepared by a method which has been shown previously to effectively isolate these proteins from *M. catarrhalis* (17). Briefly, organisms were harvested from either CDM 0 or CDM 100 broth culture by centrifugation at 1,500 × *g* for 10 min at 4°C. The wet weight of the bacteria was determined, and the organisms were suspended in 1 ml of 1 M sodium acetate with 0.001 M β-mercaptoethanol (pH 4.0) per g of bacteria. Freshly prepared 5% Zwittergent 3-14 containing 0.5 M $CaCl_2$ (pH 8.0) was added, and the suspension was vortexed. Cold ethanol was added to the supernatant to a final volume of 20%, and the mixture was centrifuged at 17,000 × *g* for 10 min at 4°C. The pellet was discarded, cold ethanol was added to a final volume of 80%, and the centrifugation step was repeated for 20 min. The pellets were air dried and resuspended in buffer Z (0.05% Zwittergent, 0.05 M Tris, 0.01 M EDTA [pH 8.0]). The protein concentration was determined by the Bio-Rad protein assay by precipitating 0.1 ml of each OMP preparation with 0.9 ml of cold ethanol overnight at −20°C. The precipitate was collected by centrifugation and allowed to air dry prior to the protein determination. Each preparation was divided into 0.1-ml aliquots and stored at −20°C. The samples were precipitated just prior to electrophoresis.

SDS-PAGE analysis and Western blot (immunoblot) assays of OMPs from *M. catarrhalis*. The OMPs from *M. catarrhalis* grown in CDM 0 and CDM 100 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (11). Briefly, 100 μg of each OMP preparation was added to each well and electrophoresed on a 7.5% separating gel with a 4% stacking gel. Each gel was run at 45 mA (constant current) until the dye front reached the bottom of the gel. The gels were stained with Coomassie blue as described previously (17). Western blot assays were performed by the method described previously (17). The blots were probed with monoclonal antibody 2.9F, which recognizes the B2 or CopB protein of *M. catarrhalis* (23), or with antiserum from a bronchiectasis patient (23).

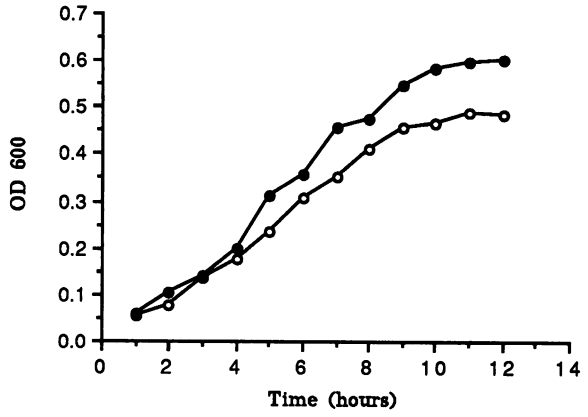

FIG. 1. Growth curves of *M. catarrhalis* 25240 in the iron-limited medium CDM 0 (○) and in medium containing 100 μM $FeNO_3$, CDM 100 (●).

RESULTS

Comparative growth rates in the presence and absence of iron. Our preliminary studies, using *M. catarrhalis* 25240, determined that an initial OD_{600} between 0.070 to 0.080 for each broth culture provided consistent results for these experiments. Figure 1 illustrates a typical growth curve of *M. catarrhalis* under iron-deficient and iron-replete conditions. Both cultures grew at similar rates for approximately 7 to 9 h. At this point, the growth rate of the bacteria in CDM 0 began to diminish, while the growth rate of the organisms in CDM 100 did not change. These data demonstrate that *M. catarrhalis* 25240 can use ferric nitrate as an iron source for growth. The data suggest that the lack of available iron limited the growth of the bacteria in the CDM 0 culture. These studies were performed with *M. catarrhalis* 56, 4223, and 1084 with similar results (data not shown).

Siderophore determinations. To assess whether *M. catarrhalis* makes siderophores in response to iron stress, strain 25240 was grown to the mid-log phase in CDM 0 and CDM 100. Supernatant from each culture was filter sterilized and analyzed by the CAS assay (22). Three separate assays failed to detect the presence of iron-binding compounds in the broth culture supernatant of organisms grown under iron-limiting conditions. These data suggested that *M. catarrhalis* does not produce siderophores in response to iron limitation (data not shown).

Growth with iron-saturated transferrin and lactoferrin. To determine if *M. catarrhalis* can utilize other iron sources for growth, iron-saturated human transferrin and lactoferrin were added to broth cultures of organisms grown in CDM 0. Figure 2 demonstrates that *M. catarrhalis* 25240 can obtain iron from human transferrin (Fig. 2A) and from human lactoferrin (Fig. 2B). These data also show that the concentration of these carrier proteins appears to be the limiting factor in the growth rate. As can be seen from each graph, organisms grown in media containing 10 μM transferrin (Fig. 2A) or lactoferrin (Fig. 2B) maintained a better growth rate than *M. catarrhalis* cultured with 2.5 μM iron carrier. Growth rates in the presence of 1.25 μM iron carrier were similar to the CDM 0 growth rates (data not shown). Coupled with our inability to detect sid-

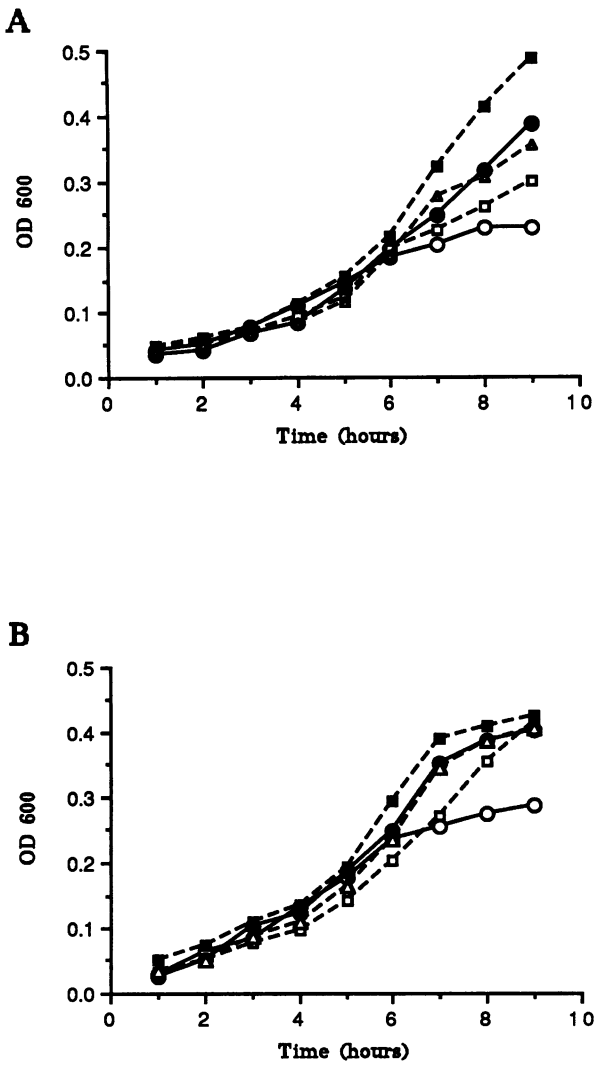

FIG. 2. Growth curves of *M. catarrhalis* 25240 in completely defined media containing iron-loaded human transferrin (A) and iron-loaded human lactoferrin (B). The concentrations of each protein are 2.5 (□), 5 (△), and 10 (■) μM. The controls on each graph are CDM 0 (○) and CDM 100 (●).

erophore production by these organisms, these results suggest that *M. catarrhalis* express specific proteins capable of binding transferrin and lactoferrin, similar to *Neisseria meningitidis* and *Neisseria gonorrhoeae*.

Proteins expressed under iron limitation. Bacteria were harvested by centrifugation after growth in iron-limited or iron-replete media. Figure 3A shows that OMPs were isolated from organisms after 10 to 12 h of growth (arrow). Figure 3B illustrates that *M. catarrhalis* 25240 grown under iron-limiting conditions (lane D) expressed proteins which were not present in the outer membranes of organisms grown in CDM 100 (lane E). At least four new proteins (numbered arrows) are evident in the outer membranes from iron-limited organisms which were not detected in the bacteria grown with an iron source. The apparent molecular masses of these proteins expressed by *M. catarrhalis* 25240 are as follows: protein 1, 115 kDa; protein 2, 110 kDa; protein 3, 108 kDa; and protein 4, 81 kDa. Experiments with *M. catarrhalis* 56, 4223, and 1084 revealed that these strains express similar proteins under iron-limiting conditions (data not shown).

Another interesting observation, noted in Fig. 3B, is the apparent increase in the relative amount of one of the major proteins (marked with an asterisk) in the outer membrane from iron-starved bacteria (lane D) compared with that from bacteria from the iron-replete culture (lane E). The apparent molecular mass of this protein (80 kDa) suggests that this may be the previously described CopB (OMP B2) protein of *M. catarrhalis* (7, 16). Studies by Helminen et al. have shown that this protein is conserved among strains of *M. catarrhalis*, and antibodies directed to this protein may be important in the pulmonary clearance of *M. catarrhalis* in a mouse model system (7).

Proteins expressed by *M. catarrhalis* 25240 under iron limitation are not induced by heat stress or growth phase. The SDS-PAGE OMP profiles shown in Fig. 3B were prepared from *M. catarrhalis* 25240 in the late-log to stationary phase of growth as illustrated in Fig. 3A. To confirm that the new proteins shown in Fig. 3B resulted from iron limitation, and were not the result of general environmental stress, the following experiments were performed.

Parallel cultures were grown in CDM 0 and CDM 100 broth, at 37 and 40°C, with constant agitation. The OD_{600} was measured, and 1 ml of each bacterial culture was harvested at 1-h intervals. The cultures were monitored for 12 h. At the end of this period, OMP preparations were made from each harvested sample by scaling down the method described previously (17).

Figure 4 is a composite illustrating the growth curve of the two cultures maintained at 37°C (Fig. 4A) and a Coomassie blue-stained SDS-polyacrylamide gel of the OMPs prepared from *M. catarrhalis* 25240, harvested at different times (arrows). The SDS-polyacrylamide gel contains matched pairs of membrane proteins from bacteria grown in CDM 0 and CDM 100. Equal amounts of OMPs from CDM 0 and CDM 100 cultures were loaded into adjacent wells.

The results of this experiment show that by 2 h, and possibly earlier, *M. catarrhalis* 25240 begins to express proteins that are not present in the OMP of the bacteria grown in iron-containing media. As illustrated in Fig. 4B, these iron-repressible proteins were expressed throughout growth in CDM 0, demonstrating that these proteins were not induced as a result of stress when the organisms reached the stationary phase. The molecular weights of these proteins correspond to those described in Fig. 4B.

In the second part of this experiment, the cultures of *M. catarrhalis* 25240 maintained at 40°C were analyzed for expression of these iron-repressible proteins. As a result of the increased temperature, both the CDM 0 and CDM 100 cultures showed extremely low growth rates, as depicted in the growth curve seen in Fig. 5A. Because of the limited amount of growth over the early time points, insufficient OMPs were isolated for SDS-PAGE analysis. However, enough bacteria were obtained to isolate OMPs from each culture at 8 h (Fig. 5A, arrow). Figure 5B is an SDS-polyacrylamide gel showing the OMP patterns isolated from *M. catarrhalis* 25240 grown in CDM 0 (left lane) and CDM 100 (right lane). *M. catarrhalis* produced the new proteins only when grown in CDM 0, indicating that increased temperature did not induce the synthesis of these proteins.

Studies confirming that the proteins expressed by *M. catarrhalis* 25240 are the result of iron limitation. An experiment was designed to determine if the expression of the new proteins, synthesized under iron-limiting conditions, could be reversed by the addition of an iron source. *M. catarrhalis* 25240

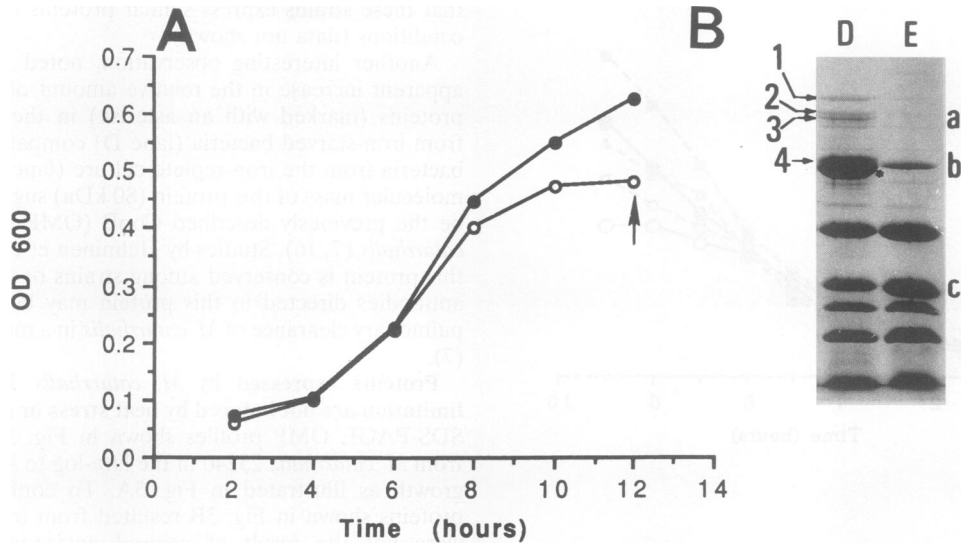

FIG. 3. (A) Growth curves of *M. catarrhalis* 25240 in CDM 0 (○) and CDM 100 (●) denoting the time point at which bacteria were harvested (arrow). (B) SDS-polyacrylamide gel of OMPs isolated from bacteria grown in CDM 0 (lane D) and CDM 100 (lane E). Molecular masses of the iron-repressible proteins are as follows: protein 1, 115 kDa; protein 2, 110 kDa; protein 3, 108 kDa; and protein 4, 81 kDa. The molecular mass standards are 105 (a), 80 (b), and 49.5 (c) kDa. The CopB or B2 protein is marked by an asterisk.

was grown in parallel cultures of CDM 0 and CDM 100 to the mid-log phase (approximately 3 h). A portion of the organisms growing in CDM 0 was used to inoculate a fresh CDM 100 culture, and growth was monitored for another 3 h. Outer membrane proteins were prepared from all three cultures and analyzed by SDS-PAGE. Figure 6 demonstrates that the proteins expressed by *M. catarrhalis* 25240, grown in an iron-limited environment, were no longer produced when these bacteria were placed into media containing an iron source. We conclude that these newly expressed proteins are iron-repressible proteins.

Western blot analysis of the iron-repressible proteins. Western blot assays were performed to begin to characterize the iron-repressible proteins expressed by *M. catarrhalis* 25240. For these studies, the bacteria were cultured on CDM 0 or CDM 100 plates and then inoculated into broth cultures as described above. Figure 7 is an enlarged composite of two Western blot assays and the SDS-polyacrylamide gel which was used to make the blots. Lanes a, c, and e contain 70 μg of OMPs isolated from *M. catarrhalis* grown in CDM 100, and lanes b, d, and f contain equal amounts of OMPs isolated from bacteria grown under iron-limiting conditions (i.e., in CDM 0). The blot on the left of Fig. 7 (in lanes a and b) was probed with monoclonal antibody 2.9F. This antibody recognizes the CopB or B2 protein of *M. catarrhalis* (7, 8, 17, 23). The blot demonstrates that the expression of this protein is increased under iron-limiting conditions.

The blot on the right of Fig. 7 (in lanes e and f) was probed with antiserum from a bronchiectasis patient with confirmed *M. catarrhalis* infection (23). This blot shows that the patient's serum contains antibodies to a protein expressed under iron limitation (CDM 0) (Fig. 7, lane f), which is not detected in the OMPs isolated from the *M. catarrhalis* grown in iron-replete media (CDM 100) (Fig. 7, lane e). These data demonstrate that this protein is iron repressible, and they further suggest that this protein is expressed during *M. catarrhalis* infection in vivo. This protein has been termed B1 (23).

The molecular masses of the proteins which were recognized by monoclonal antibody 2.9F and the patient serum were calculated from the enlargement of the SDS-polyacrylamide gel shown in the center of Fig. 7 (lanes c and d). The upper arrow denotes protein B1 with an apparent molecular mass of 81 to 82 kDa, while the lower arrow represents CopB or B2 with an apparent molecular mass of 80 kDa.

DISCUSSION

In this report, we describe the first studies which demonstrate that *M. catarrhalis* expresses iron-repressible proteins. Our data also demonstrate that these proteins are located in the outer membrane, they are not expressed as a result of general environmental stresses, and expression can be reversed by placing iron-starved bacteria into an iron-replete environment. We have also shown that *M. catarrhalis* can utilize ferric nitrate as an iron source for continued growth in vitro. Under this iron-replete condition, the iron-repressible proteins were not detected, suggesting that these proteins may be important in mechanisms which allow *M. catarrhalis* to obtain and utilize iron.

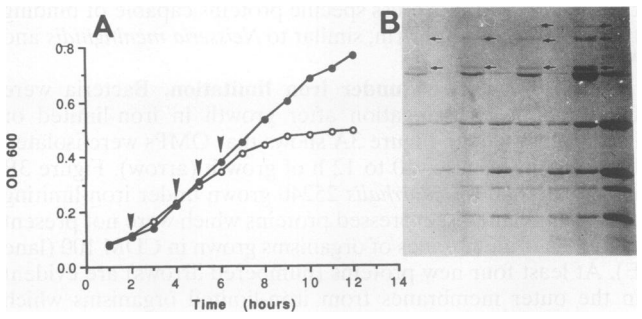

FIG. 4. (A) Growth curves of *M. catarrhalis* 25240 in CDM 0 (○) and CDM 100 (●). Bacteria were harvested at intervals during the log phase as indicated by arrowheads. (B) SDS-PAGE analysis of matched sets of OMPs prepared at each time point. Arrows denote iron-repressible proteins.

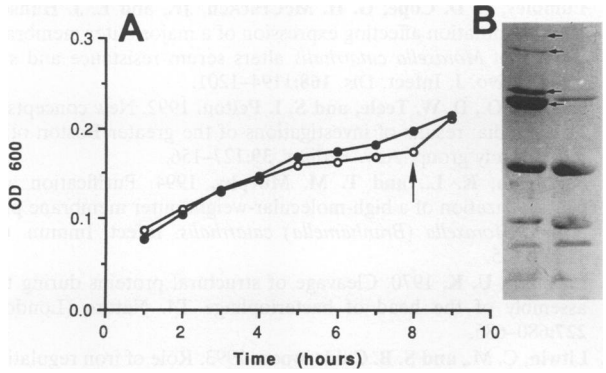

FIG. 5. (A) Growth curves of *M. catarrhalis* 25240 grown in CDM 0 (○) and CDM 100 (●) at 40°C. The arrow denotes the time point at which the bacteria were harvested. (B) SDS-PAGE analysis of OMPs isolated from bacteria cultured in CDM 0 (left lane) or CDM 100 (right lane). Arrows denote iron-repressible proteins.

Our data have also demonstrated that *M. catarrhalis* does not secrete siderophores in response to iron starvation. However, our studies have confirmed that *M. catarrhalis* can obtain the necessary iron for growth in vitro from the human carrier proteins transferrin and lactoferrin. This is not uncommon, as mechanisms have been described for *H. influenzae* and pathogenic *Neisseria* species which allow these mucosal pathogens to directly utilize host iron complexes for growth (12–14). Receptors for human transferrin and human lactoferrin have been identified on both *N. meningitidis* and *N. gonorrhoeae* (20). These proteins are expressed on the surface of these bacteria in response to iron limitation, and it is believed that these lactoferrin and transferrin receptors are essential for survival of these pathogens during in vivo infection (5). Evidence exists which suggests that similar proteins may exist on *M. catarrhalis*. Studies by Shryvers et al. have shown that *M. catarrhalis* binds human transferrin and lactoferrin in a dot blot assay (21). Furthermore, these same investigators demonstrated that crude total membranes prepared from *M. catarrhalis* contain

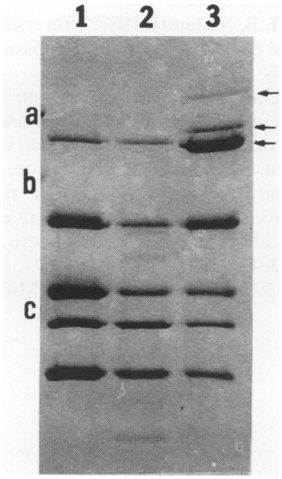

FIG. 6. SDS-polyacrylamide gel showing OMPs isolated from bacteria grown in CDM 0 (lane 3) and CDM 100 (lane 1) and from bacteria removed from CDM 0 medium and cultured in CDM 100 (lane 2). Arrows denote iron-repressible proteins. Molecular mass standards are 105 (a), 80 (b), and 49.5 (c) kDa.

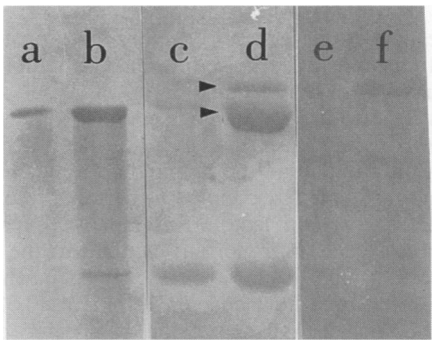

FIG. 7. A composite of two Western blot assays (lanes a and b and e and f) and an SDS-polyacrylamide gel (lanes c and d). Lanes: a, c, and e, OMPs from *M. catarrhalis* grown in CDM 100; b, d, and f, OMPs from bacteria grown in CDM 0. Lanes a and b were probed with monoclonal antibody 2.9F, and lanes e and f were probed with antiserum from a bronchiectasis patient. Lanes c and d are the SDS-polyacrylamide gel showing the molecular masses of the proteins which react in each blot. The apparent molecular masses are 81 to 82 (upper arrow) and 80 (lower arrow) kDa.

proteins that bind to human transferrin and lactoferrin (21). It should be noted that the apparent molecular weights of the proteins described in these studies (21) are similar to the those of the iron-repressible proteins shown in Fig. 3. Because *M. catarrhalis* is closely related to the pathogenic *Neisseria* species, it is not surprising that this human pathogen may have evolved similar mechanisms of iron acquisition which allow these organisms to survive and replicate on mucosal surfaces.

Our studies have also identified specific proteins which are expressed in the outer membranes of *M. catarrhalis* grown under iron-limiting conditions. Although the functions for the proteins are unknown, it appears that at least two of these proteins may be important during infections with *M. catarrhalis*. A recent report describes the identification and isolation of a protein present in the outer membrane of multiple clinical isolates of *M. catarrhalis* (23). This protein has an apparent molecular mass of 81 kDa, and it has been demonstrated that patients with bronchiectasis and chronic colonization with *M. catarrhalis* have antibody directed to this protein (23). This protein has been termed B1, and it is distinct from the CopB (OMP B2) protein described previously (8, 17). Our data (Fig. 1B), derived by using the antiserum used previously to detect this protein (Fig. 7), show that the B1 protein of *M. catarrhalis* is expressed under iron-limiting conditions, suggesting a possible role in mechanisms of iron acquisition or utilization. However, further studies are needed to define the function of the B1 protein.

The CopB (OMP B2) protein, described by Helminen et al., is an OMP conserved in many strains of *M. catarrhalis* and has an apparent molecular mass of 80 kDa (8, 16). A monoclonal antibody directed to CopB enhances the pulmonary clearance of *M. catarrhalis* in a mouse animal model (7). Furthermore, a CopB-deficient mutant of *M. catarrhalis* is sensitive to serum killing and does not grow well in the lungs of mice, unlike the parent strain which expresses CopB (8). Although these studies suggest the importance of this protein, the function of the CopB protein is as yet undefined. In our present study, a major OMP of *M. catarrhalis* 25240, with an apparent molecular mass of 80 kDa, shows markedly increased expression under iron-limiting conditions. On the basis of molecular weight and recent monoclonal antibody analyses, using monoclonal antibody 2.9F, we conclude that this is the CopB protein described

previously (8, 16, 23). Although these findings are consistent with those concerning other iron-repressible proteins, more detailed studies are needed to determine whether the CopB protein functions in iron acquisition.

In this study, we have identified specific proteins expressed by *M. catarrhalis* in response to iron limitation. There is very little free iron available in the human body since much of the extracellular iron is avidly coupled to carrier proteins such as transferrin (serum) and lactoferrin (mucosal surfaces) (12). Because iron is such an important element to the survival of most microbes, many bacteria have evolved mechanisms for obtaining iron from their host environment (3, 25). These mechanisms, such as the expression of specific proteins or receptors, are often linked to virulence since they allow these human pathogens to survive and grow in vivo. The data we have presented in this report demonstrate that *M. catarrhalis* can compete for iron complexed to either human transferrin or lactoferrin. Furthermore, *M. catarrhalis* expresses specific proteins in response to growth under iron-limiting conditions in vitro. These results suggest that these new proteins may serve as specific receptors for iron acquisition. More extensive studies are warranted to determine the role of these iron-repressible proteins in the pathogenesis of *M. catarrhalis* infections.

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